Environmental Health Perspectives
Vol. 93, 97–103, 1991

Mechanisms of Oncogene Cooperation: Activation and Inactivation of a Growth Antagonist

by Michelle M. Ragozzino,* Alan Kuo,* James DeGregori,* Nancy Kohl,*† and H. Earl Ruley*

Gene transfer experiments have defined limitations with regard to the ability of individual oncogenes to transform cultured cells to a tumorigenic state. The stable transformation of REF52 cells by either the *ras* or *sis* oncogenes requires the continuous expression of a second collaborating oncogene, such as adenovirus-5 E1A or SV40 large T-antigen. Our studies suggest that the function of the nuclear collaborators is to antagonize dominant growth controls which limit the ability of REF52 cells to proliferate in response to mitogenic stimuli.

Introduction

Oncogenes are genes implicated in carcinogenesis by virtue of their associations with oncogenic viruses and tumor-specific chromosome abnormalities and by their ability to transform cultured cells to a tumorigenic state (*1,2*). Mutations and virus associations enable oncogenes to facilitate autonomous cell growth either by increasing the expression of activities that promote cell proliferation or by interfering with controls that normally serve to restrict cell growth. As carcinogenesis is generally believed to be a multistep process, malignant phenotypes may frequently require multiple alterations affecting several levels of growth control (*3*). This is supported by gene-transfer experiments in which two or more oncogenes are required to transform normal cells to a malignant state (*4–6*).

Collaborations between oncogenes indicate that regulatory mechanisms that preclude transformation by one oncogene can be circumvented by a second oncogene. This implies that certain oncogenes actively influence the way that cells respond to other oncogenes. Gene transfer experiments indicate that expression of an activated *ras* oncogene is sufficient to transform cells from a variety of established cell lines (*7–9*). In contrast, primary and early passage rodent cells are transformed by *ras* oncogenes only at very low frequencies unless cotransfected with collaborating genes such as E1A or *myc* (*9,10*).

Several observations suggested that *in vitro* establishment is a necessary prerequisite to transformation by *ras* oncogenes. First, the susceptibility of normal hamster cells to transformation by *ras* coincides with *in vitro* establishment (*9*). Second, genes such as E1A, p53, SV40 large T-antigen, and *myc*, which collaborate with *ras* to transform primary cells, also facilitate immortalization (*11–15*). Finally, mutational analysis of E1A revealed a tendency for mutations that affect *in vitro* establishment functions to also influence the ability of E1A to collaborate with *ras* in the transformation of primary cells (*16,17*). However, some E1A genes are defective in immortalization but are nevertheless able to collaborate with *ras* in transformation (*17–19*).

Though informative, the use of primary cells in transformation studies has several limitations. First, a variety of factors including variations in cell type, levels of *ras* expression, inhibitory effects of normal cells, and other environmental factors can influence transformation by *ras* alone (*20–24*). Second, the fact that immortalization appears to be a necessary prerequisite for oncogenic transformation *in vitro* may simply reflect difficulties imposing a transformed phenotype over a dominant commitment to senesce. Finally, problems working with nonestablished cell clones complicate any assessment of the phenotypic effects of *ras* alone. As a result, the mechanisms responsible for oncogene cooperation in nonestablished cells have been difficult to analyze.

*Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139.

†Present address: Merck, Sharp, and Dohme Research Labs, West Point, PA 19486.

Address reprint requests to H.E. Ruley, Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139.

We have examined oncogene interactions using an established line of Fisher rat embryo fibroblasts, REF52, which, unlike most established lines, is not stably transformed by activated *ras* oncogenes. Instead, transformation of REF52 cells by *ras* strictly requires the presence of a collaborator, such as adenovirus E1A or SV40 large T-antigen (*25*). The transformation properties of the REF52 line indicate that the role of the viral oncoproteins extends beyond that of promoting establishment and suggests that while immortality may be a necessary prerequisite for transformation by *ras*, it is not sufficient. While other established cell lines resist transformation by *ras* (*14,26*), the REF52 line provides the clearest evidence that oncogenes can complement in a genetic sense to elicit a malignant phenotype.

Multistep Transformation of REF52 Cells

In earlier studies, REF52 cells were transfected with T24-Ha-*ras* linked to the neomycin-resistance gene and clones were selected in G418. The vast majority of the resistant clones were morphologically indistinguishable from the parental line, unable to grow in soft agar, not tumorigenic in nude mice, and expressed low levels of $p21^{T24Ha\text{-}ras}$ (30% of the level of endogenous Ha-*ras* p21). While 1 in 1000 clones was morphologically transformed, these rare *ras*-only transformants experienced morphological crisis and ceased growing. $p21^{T24Ha\text{-}ras}$ levels in these abortive transformants were extremely high (greater than 100-fold higher than the endogenous Ha-*ras* p21), indicating that even high levels of the activated *ras* oncoprotein are not sufficient to induce stable transformation in the absence of a collaborating oncogene (*25*).

In contrast, cells transfected with T24Ha-*ras/neo* and either adenovirus E1A or SV40 large T-antigen, gave rise to transformed neo-resistant colonies (*25,27*). While $p21^{T24Ha\text{-}ras}$ levels in these transformed clones varied considerably, ranging from levels 2- to 10-fold greater than the endogenous $p21^{c\text{-}Ha\text{-}ras}$, all of the transformed clones tested formed colonies in soft agar and induced rapidly lethal tumors in nude mice. These studies indicate that relatively low levels of the oncogenic p21 are sufficient to transform in the presence of a collaborator and that the collaborating oncogene supplies activities that are not duplicated by increased *ras* expression.

Conditional transformation of REF52 cells has also been achieved by cotransfecting a temperature-sensitive allele of SV40 large T-antigen (*tsA*58) and T24 Ha-*ras* (*27*). Thus, two-thirds of the clones transfected with T24 Ha-*ras* and *tsA* ceased growing, arresting predominantly in G_2 or late S-phase, when transformed from a permissive to a nonpermissive temperature for T-antigen expression. $p21^{T24\ Ha\text{-}ras}$ levels were unaffected by the temperature shift. This study indicates that transformation by *ras* requires continuous expression of a collaborating oncogene, and suggests that E1A and SV40 large T-antigen may enable *ras* to transform REF52 cells by circumventing cellular responses to *ras* which inhibit cell proliferation.

Oncogenic p21 Induces Quiescent REF52 Cells to Proliferate

To investigate early phenotypic effects of *ras*, activated and normal forms of p21 were introduced into serum-arrested REF52 cells using the bead-loading method (*28*). The method utilizes small (75–500 μm) glass beads to mechanically disrupt the plasma membrane to an extent that allows proteins to enter cells without significantly affecting cell viability. $p21^{Ha\text{-}ras}$ proteins were purified from insect cells infected with baculovirus vectors expressing high levels of posttranslationally modified T24 and c-Ha-*ras* p21s. As shown in Figure 1, up to 31% of the cells loaded in the presence of a 2.2 mg/mL solution of the activated p21 were stimulated to enter S phase within 24 hr, whereas cells loaded with bovine serum albumin failed to respond. Introduction of nonactivated c-Ha-*ras* p21 was less effective in stimulating cell cycle progression than activated p21 (data not shown). Maximum incorporation of ^{3}H-thymidine was observed 12 to 24 hr after exposure to bead loaded $p21^{T24Ha\text{-}ras}$. At concentrations greater

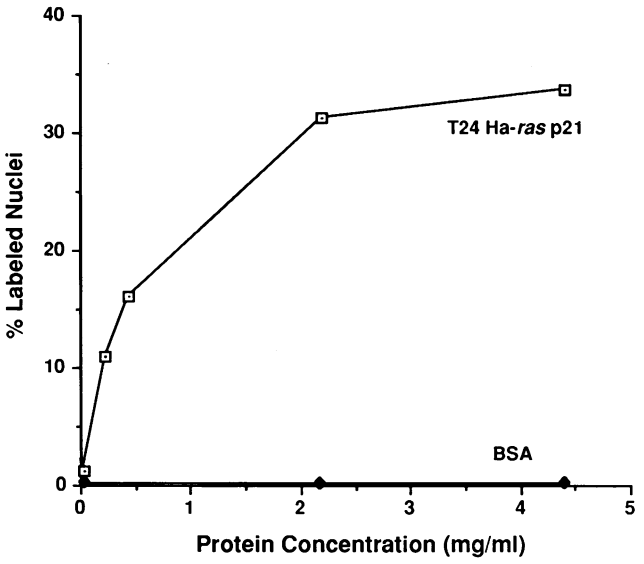

FIGURE 1. Bead-loaded T24*ras* p21 stimulates serum-arrested REF52 cells to progress through the cell cycle. REF52 cells, seeded on glass coverslips, were transferred to Dulbecco's modified Eagle's media containing 1% ITS+ (insulin, transferrin selenium, and oleic acid/bovine serum albumin complex). After 48 hr, growth-arrested REF52 cells were washed with phosphate-buffered saline (PBS), covered with 50 μL PBS containing varying amounts of T24*ras* p21, or bovine serum albumin (BSA), and were bead-loaded using 75- to 150-μm beads, as described (*28*). BSA loaded at 3.6 mg/mL resulted in 0.1% labeled nuclei. Cells were labeled with 2.5 μCi/mL ^{3}H- thymidine for 24 hr post-bead-loading and then fixed and processed for emulsion autoradiography.

than 1 mg/mL, $p21^{T24Ha\text{-}ras}$ also induced morphological transformation as well as stimulating DNA replication.

Similar results have been reported using microinjected p21 (*29*); thus, although resistant to transformation, REF52 cells are mitogenically stimulated by introduction of $p21^{T24Ha\text{-}ras}$. In this regard, REF52 cells respond to oncogenic p21 in a manner not unlike cells that are susceptible to transformation (*29,30*). As *ras* is thought to transduce signals from growth factor receptors, controls that prevent *ras* from transforming REF52 cells would appear to act at a level subsequent to or independent of a mitogenic response to *ras*.

E1A Enables the *sis* Oncogene to Transform REF52 Cells

Similar growth controls may also regulate transformation of REF52 by c-*sis*, the B chain of the platelet-derived growth factor (PDGF) (*31,32*). Transformation by *sis* is thought to result from autocrine stimulation resulting from binding to the growth factor to PDGF receptors.

Quiescent REF52 cells were stimulated to enter S phase by recombinant $p28^{c\text{-}sis}$, indicating that these cells have receptors for PDGF (Fig. 2). In addition, $p28^{c\text{-}sis}$ induced, with expected kinetics, the transcription of several growth factor-induced early response genes (i.e., *myc*, *fos*, and JE), suggesting that the signal transduction pathway from the cell surface to the nucleus is intact (data not shown). However, when REF52 cells are cotransfected with c-*sis* and the gene for neomycin resistance, all of the resistant colonies were indistinguishable from REF52 cells transfected with the neomycin-resistance gene alone. In each case, similar numbers of neo-resistant colonies were obtained, suggesting that the *sis* gene was not toxic to REF52 cells. E1A, introduced together (Table 1) or in a secondary transfection (data not shown), enabled c-*sis* to transform REF52 cells to anchorage independence.

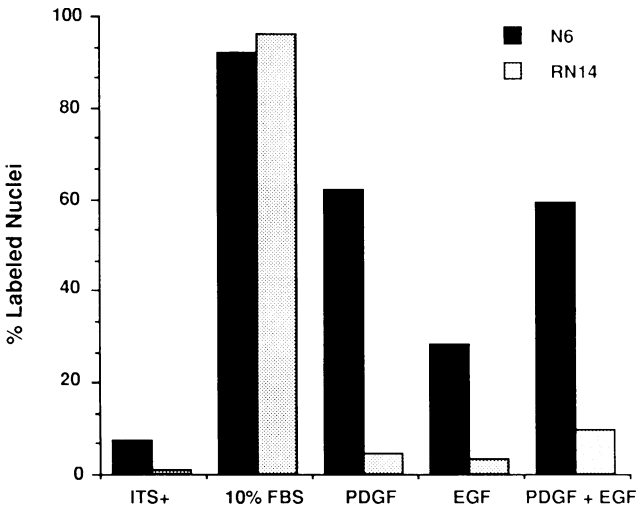

FIGURE 2. T24 Ha-*ras* p21 inhibits cellular responses to mitogenic stimuli. REF52 cells expressing *neo* (N6) or T24 Ha-*ras* genes (RN14) were seeded on cover slips at a density of 400 cells/cm^2 and grown for 48 hr. At this time, monolayers were washed twice with phosphate-buffered saline and transferred to Dulbecco's modified Eagle's medium ITS+ (insulin, transferrin selenium, and oleic acid/bovine serum albumin complex). After 48 hr, the indicated growth factors were added to the G_0-arrested cells. Samples were labeled for 12 hr at 12-hr intervals with 5 μ Ci/mL ^{3}H-thymidine and then processed for emulsion autoradiography. Results for the time period 12 to 24 hr after growth factor addition (i.e., the interval with maximum response) are plotted. The growth factor concentrations were as follows: serum (10% fetal calf); recombinant platelet-derived growth factor B-chain (PDGF, 40 ng/mL); epidermal growth factor (EGF 100 ng/mL). RN14 cells also failed to respond to phorbol 12-myristate 13-acetate (TPA) at 10 ng/mL (data not shown).

These results suggest that the ability of cells to be mitogenically stimulated by *sis* growth factor, while perhaps necessary, was not sufficient for stable transformation. As with *ras*, E1A appears to circumvent controls that prevents sustained exposure to a mitogenic stimulus from transforming.

Collaborating Oncogenes Have Complementary Effects on Cellular Responses to Growth Factors

Since oncogenic transformation of REF52 cells can require the activities of two oncogenes acting in concert, these cells provide an opportunity to study the biological effects of individual oncogenes in the absence of secondary phenotypic changes resulting from transformation. REF52 cells expressing low levels of activated Ha-*ras* p21 (T24REF) are morphologically normal and fail to form either colonies in soft agar or tumors in nude mice. However, these cells express functional *ras* oncoproteins, since E1A and SV40 large T-antigen each efficiently transform when transfected subsequently (clone N6) (*25*).

When transferred to serum-free media, T24REF (clone RN14) cells cease growing with kinetics indistinguishable from the neo-resistant REF52 control (clone N6) (Fig. 3). Nevertheless, a marked difference was evident between the cell lines upon restimulation by individual mitogens. As shown in Figure 2, the presence of even low levels of activated p21 interfered with the ability of REF52 cells to progress into S phase upon stimulation by phorbol 12-myristate 13-acetate, epidermal growth factor, PDGF or PDGF and epidermal growth factor together. The response to serum, however, was unaffected or even moderately enhanced by T24Ha-*ras*.

Cell lines individually expressing either E1A or SV40 large T antigen, although morphologically altered, were not transformed, as assessed by their failure to grow in soft agar or from tumors in nude mice. However, as shown in Figure 2, the presence of either SV40 large T antigen or E1A in REF52 cells allows for extended proliferation in the absence of serum growth factors. Thus, there is an apparent functional complementation: *ras* inhibits proliferative responses to mitogenic stimuli, while E1A and large T each enable REF52 cells to proliferate in the absence of mitogenic stimuli. In other words, REF52 cells appear to possess growth controls

Table 1. *Sis* and adenovirus-5 E1A cooperate to transform REF52 cells.

Plasmid	Transfected genes	Experiment no.	Selection	Soft agar plating efficiency
Homer6	*neo*	1	G418	< 0.005
		2	G418	< 0.005
pSMR1MoNeo	*sis, neo*	1	G418	< 0.005
		2	G418	< 0.005
p1Ahgm	Ad5 E1A, *hph*	1	Hygromycin	< 0.005
		2	Hygromycin	< 0.005
pSMR1MoNeo		1	G418	0.6
+ p1A	Ad5 E1A, *hph, sis*	2	G418	0.2
		3	G418	0.2
		4	G418	0.1
pSMR1MoNeo	Ad5 E1A, *neo, sis, hph*	1	G418 and	5.0
+ p1Ahmg		2	hygromycin	0.2

[a]REF52 cells, 2×10^5, were transfected with the indicated plasmids containing the following genes: Homer6, a neomycin (G418) resistance gene (*20*), pSMR1MoNeo, a derivative of pSM1 (*42*), c-*sis* and *neo* genes expressed from the SV40 early promoter and moloney murine leukemia virus LTR, respectively; p1A, advenovirus-5 E1A (*25*); and p1Ahgm, Ad5 E1A and hygromycin resistance gene. Following selection with 0.4 mg/mL G418 (Gibco, 50% active) and/or 0.1 mg/mL hygromycin B (Calbiochem), cells from at least 100 colonies were trypsinized, pooled, and plated in media containing 0.37% agarose. Fresh agar-containing media was added to soft agar cultures once a week for 3 to 4 weeks and then the cultures were stained with *p*-iodonitrotetrazolium violet (Sigma). Soft agar plating efficiency refers to percent of cells forming colonies 70 μm in diameter or larger.

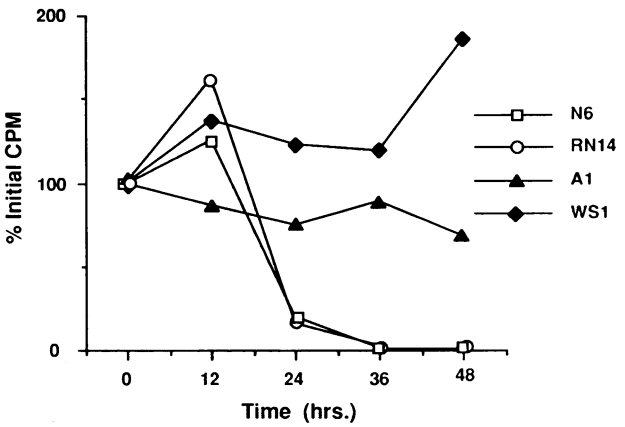

FIGURE 3. REF52 cells expressing adenovirus E1A or SV40 large T-antigen continue to proliferate in serum-free media. Cell lines were seeded at a density of 3000 cells per 35 mm well and allowed to attach and proliferate for 48 hr. Cells were then washed twice with phosphate-buffered saline and transferred to media containing Dulbecco's modified Eagle's medium (DME) and 1% ITS+ (insulin, transferrin selenium, and oleic acid/bovine serum albumin complex). This is the zero time point. Cells were labeled for 12 hr prior to each of the indicated time intervals in 1 mL of DME/ITS+ containing 5 μ Ci ^{3}H-thymidine. Trichloroacetic acid-precipitable counts were determined and expressed as a percent of the time zero (i.e., label added from −12 to 0 hr) cpm. Results with REF52 clones expressing the following genes are plotted: *neo*, N6; T24 Ha-*ras*, RN14; adenovirus-5 E1A, A1; and SV40 large T-antigen, WS1.

which limit the ability of the cells to proliferate in response to mitogenic stimuli. These controls are activated by *ras* and are circumvented by E1A and SV40 large T-antigen.

A Dominant Antagonist Regulates Transformation by *ras* and E1A

A series of cell fusion experiments were performed to determine whether the growth controls that prevent *ras* from transforming REF52 cells are dominant or recessive. In principle, REF52 could differ from other established rodent lines, which are transformed by *ras* alone, in one of two ways: *a*) REF52 cells lack activities that are required for transformation by *ras*, and E1A supplies the missing cellular functions; *b*) REF52 cells possess an antagonist that prevents *ras* from transforming, and E1A inactivates of circumvents the antagonist. In order to distinguish between these two possibilities, cell lines, shown in Table 2, containing either hygromycin or neomycin resistance markers, were fused using polyethylene glycol and selected in the presence of both antibiotics (*33*). Hybrid colonies were pooled and plated in soft agar to assess whether any expressed transformed phenotypes (Table 3).

When NRK cells transformed by *ras* were fused with REF52 cells, 20-fold fewer colonies were obtained than when normal NRK cells were fused with REF52. Furthermore, the hybrid colonies obtained were morphologically normal and failed to produce any soft agar colonies, indicating that transformation of NRK cells by *ras* was suppressed by fusion with REF52 cells. In contrast, the fusion of *ras*-transformed NRK cells with E1A-expressing REF52 cells yielded transformed colonies, which formed colonies in soft agar with a frequency of 4%. Similar results were obtained with Rat2 cells were substituted for NRK cells (Table 3). Thus, the growth controls that prevent *ras* from transforming and act to inhibit proliferation in the presence of *ras* are dominant, *trans*-acting, and are either circumvented or negated by function(s) of E1A.

Further evidence that REF52 cells possess a dominant antagonist of transformation has come from the analysis of spontaneous transformants induced by activated *ras* oncogenes. We pooled 10^2 to 10^4 to neomycin-resistant colonies isolated after transfecting REF52 cells with T24Ha-*ras*, and 5×10^6 cells were injected into nude mice. Tumors arose within 4 to 6 weeks, and the cell lines derived from each tumor were

Table 2. Cell lines used as fusion partners.[a]

Cell line	Plasmid	Transfected gene(s)	Selection	Morphology	Soft agar plating efficiency
REF52.H1	pY3	*hph*	hyg^R	Normal	< 0.01
REF52.E1aH1	p1Ahgm	E1A, *hph*	hyg^R	Normal	< 0.01
Rat2.H1	pY3	*hph*	hyg^R	Normal	< 0.01
Rat2.G1	Homer6	*neo*	neo^R	Normal	< 0.01
Rat2.T24G3	pT24neo	*ras, neo*	neo^R	Transformed	34
NRK49F.H1	pY3	*hph*	hyg^R	Normal	0.2
NRK49F.T24G3	pT24neo	*ras, neo*	neo^R	Transformed	10
N5 (REF52)	pKOneo	*neo*	neo^R	Normal	0.02
RT1	pT24neo	*ras, neo*	neo^R	Transformed	1
RC6T1	HO6T1	*ras, neo*	neo^R	Transformed	3
RC6T2	HO6T1	*ras, neo*	neo^R	Transformed	0.6
15-26-4	HO6T1	*ras, neo*	neo^R	Transformed	5

[a]REF52, Rat2, and NRK49F cells were transfected with the indicated plasmids. Plasmids expressed the following genes: pT24neo, an activated Ha-*ras* gene derived from T24 human bladder carcinoma cells (*25*); p1Ahgm, the adenovirus 5 E1A gene linked to *hph*, the gene of hygromycin B phosphotransferase; Homer6, *neo* (*20*); pY3, *hph* (*43*); HO6T1, T24 H-*ras* inserted into Homer6 (*20*); and pSVrasC, a T24 *ras* cDNA replacing the *neo* of pKOneo.

Table 3. Resistance of REF52 cells to transformation by *ras* is dominant over susceptibility to transformation.[a]

Neo^R parent	× Hyg^R parent	Hybrids 10^5 cells fused	Soft agar plating efficiency
NRK49F.T24G3	REF52.H1	11 ± 5	< 0.003
	NRK49F.H1	211 ± 14	4 ± 3
	Rat2.H1	209 ± 7	4 ± 4
	REF52.E1aH1	28 ± 15	2 ± 1
Rat2.T24G3	REF52.H1	28 ± 18	0.04 ± 0.05
	NRK49F.H1	121.3 ± 0.9	1.6 ± 0.3
	Rat2.H1	175 ± 11	9 ± 2
	REF52.E1aH1	20 ± 5	17 ± 4

[a]Cells were fused with polyethylene glycol as described (*33*). After 9 to 12 days of selection in media containing 400 μg/mL G418 and 100 μg/mL hygromycin B, hybrid colonies were counted, trypsinized, and plated in 0.37% soft agarose. The soft agar plating efficiency refers to the percent of cells forming viable colonies after 3 weeks. NRK49F.T24G3 and Rat2.T24G1 are NRK49F and Rat2 cell lines transformed by T24 Ha-*ras*, respectively. Means and SEs for at least two experiments are presented.

Table 4. Spontaneous transformation of REF52 cells by *ras* typically results from recessive changes.[a]

Neo^Rparent	× Hyg^R parent	Hybrids/10^5 cells fused	Soft agar plating efficiency
RT1	REF52.H1	11	< 0.01
	NRK49F.H1	16	0.2
	Rat2.H1	76	0.5
	REF52.E1aH1	148	3
RC6T1	REF52.H1	1	< 0.01
	NRK49F.H1	68	6
	Rat2.H1	51	6
	REF52.E1aH1	6	ND
15-26-4	REF52.H1	0.5	< 0.01
	NRK49F.H1	53	7
	Rat2.H1	89	3
	REF52.E1aH1	2	0.02
RC6T2	REF52.H1	4	0.03
	NRK49F.H1	113	1.0
	Rat2.H1	9	0.01
	REF52.E1aH1	9	0.04
N5 (REF52)	REF52.H1	62	0.005
	NRK49F.H1	9	ND
	Rat2.H1	18	< 0.005
	REF52.E1aH1	35	< 0.005

[a]*ras*-Transformed REF52 tumor lines (RT1, RC6T1, RC6T2, and 15-26-4) were fused with the indicated hyg^R cells. Cell lines and methods are described in Tables 1 and 2 and in text. Fusions between REF52 cells and *ras*-transformed REF52 cells isolated by *in vivo* selection failed to produce transformed hybrids. ND, not determined.

re-established in culture and used in fusion experiments (Table 4). Fusions between tumor cells and the parental REF52 cells failed to produce transformed hybrids, whereas fusions between tumor cells and NRK or Rat2 lines yielded transformed colonies which grew in soft agar. In addition, fusions between some of the tumor lines and REF52 cells expressing E1A produced transformed hybrids, indicating that the antagonist was neutralized by E1A. This series of fusions suggest that the *ras*-induced tumors arose as a result of the loss of antagonist functions which normally prevent transformation and not from the activation of cellular functions analogous to E1A.

While the biochemical mechanisms are yet unknown, this genetically defined antagonist resembles an anti-oncogene, a gene whose functional loss leads to transformation. This is provocative, since the ability of E1A proteins to bind the retinoblastoma gene product, $p105^{Rb}$, is tightly linked to E1A activities that enable *ras* to transform primary baby rat kidney cells (*34–36*). This raises the possibility that $p105^{Rb}$ is a component of the transformation antagonist of REF52 cells. If so, one would expect to observe differences in the levels of $p105^{Rb}$ in normal REF52 cells, and *ras*-transformed REF52 tumor cells. However, the eight *ras*-induced tumor lines examined thus far maintain a normal number of intact *Rb* genes as well as unaltered levels of *Rb* message. These results do not exclude the possibility

of point-mutations or posttranslational alterations that adversely affect the activity of $p105^{Rb}$.

Summary

In summary, REF52 cells possess growth controls that prevent a sustained mitogenic stimulus from transforming. These growth controls are dominant and can act in *trans* to antagonize transformation of rat cells previously transformed by *ras*. Given that *ras* and *sis* initially provoke REF52 cells to proliferate, the antagonist may itself be activated by mitogens as a part of a feed-back mechanism which limits cell proliferation. E1A and SV40 large T are able to circumvent or inactivate the antagonist. The extent to which this relates to the mechanism by which the viral oncoproteins enable REF52 cells to grow in the absence of serum is currently under investigation.

Future studies will focus on genes, such as *Rb* and p53, known to be involved in the negative regulation of cell proliferation in an effort to understand at a biochemical level the information obtained in genetic analyses. In addition, we will examine the effects of *ras* on various cell-cycle genes. It is interesting that *ras* causes REF52 and other cells to arrest in G_2 upon removal of SV40 large T-antigen (*27,37*). The control of the cells cycle in G2 has been described in a variety of organisms and can involve a protein kinase identified as: *a*) a component of maturation promoting factor (MPF), *b*) a 34 kDa protein in mammalian cells, *c*) a starfish histone M-phase kinase, and *d*) products of the CDC2 and cdc28 genes of *S. pombe* and *S. cerevisiae*, respectively (*38,39*). That E1A both induces p34 in baby rat kidney cells (*40*) and binds to a cyclinlike molecule (*41*) as well as the observation that *ras*-expressing REF52 cells arrest in G2 suggests that p34 may influence complementation between these oncogenes. For example, E1A may protect against the repression of p34 expression or activity by *ras*.

This work is supported by National Cancer Institute Public Health Service grants R01CA40602 and P01CA42063 (to H.E.R.) and partially supported by a Cancer Center Core grant P30CA14051 to P.A. Sharp.

REFERENCES

1. Bishop, J. M. The molecular genetics of cancer. Science 235: 305–311 (1987).
2. Friend, S. H., Dryja, T. P., and Weinberg, R. A. Oncogenes and tumor-suppressing genes. N. Engl. J. Med. 318: 618–622 (1988).
3. Pitot, H. C. Fundamentals of Oncology. Marcel Dekker, Inc., New York, 1986.
4. Weinberg, R. A. The action of oncogenes in the cytoplasm and nucleus. Science 230: 770–776 (1985).
5. Ruley, H. E. Analysis of malignant phenotypes by oncogene complementation. In: Advances in Viral Oncology (G. Klein, Ed.), Plenum Press, New York, 1987, pp. 1–20.
6. Ruley, H. E. Transforming collaborations between *ras* and nuclear oncogenes. Cancer Cells 2: 258–268 (1990).
7. Goldfarb, M., Shimizu, K., Perucho, M., and Wigler, M. Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells. Nature 296: 404–409 (1982).
8. Capon, D. J., Chen, E. Y., Levinson, A. D., Seeberg, P. H., and Goeddel, D. V. Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. Nature 302: 33–37 (1983).
9. Newbold, R. F., and Overell, R. W. Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-*ras* oncogene. Nature 304: 648–651 (1983).
10. Land, H., Parada, L. F., and Weinberg, R. A. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304: 596–602 (1983).
11. Houweling, A., Van der Elsen, P. J., and Van der Eb, A. J. Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. Virology 105: 537–550 (1980).
12. Rassoulzadegan, M., Naghashfar, Z., Cowie, A., Carr, A., Grisoni, M., Kamen, R., and Cuzin, F. Expression of the large T protein of polyoma virus promotes the establishment in culture of "normal" rodent fibroblast cell lines. Proc. Natl. Acad. Sci. U.S.A. 80: 4354–4358 (1983).
13. Ruley, H. E., Moomaw, J. F., and Maruyama, K. Avian myelocytomatosis virus *myc* and adenovirus early region 1A promote the *in vitro* establishment of cultured primary cells. In: Cancer Cells 2: Oncogenes and Viral Genes (G. F. Vande Woude, A. J. Levine, W. C. Topp, and J. D. Watson, Eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1984, pp. 481–486.
14. Jenkins, J. R., Rudge, K., and Currie, G. A. Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. Nature 312: 651–564 (1984).
15. Mougneau, E., Lemieux, L., Rassoulzadegan, M., and Cuzin, F. Biological activities of v-*myc* and rearranged c-*myc* oncogenes in rat fibroblast cells in culture. Proc. Natl. Acad. Sci. U.S.A. 81: 5758–5762 (1984).
16. Zerler, B., Moran, B., Maruyama, K., Moomaw, J., Grodzicker, T., and Ruley, H. E. Adenovirus E1A coding sequences that enable *ras* and *pmt* oncogenes to transform cultured primary cells. Mol. Cell. Biol. 6: 887–899 (1986).
17. Schneider, J. F., Fisher, F., Goding, C. R., and Jones, N. C. Mutational analysis of the adenovirus E1A gene: the role of transcriptional regulation in transformation. EMBO J. 6: 2053–2060 (1987).
18. Jochemsen, A. G., de Wit, C. M., Bos, J. L., and van der Eb, A. J. Transforming properties of a 15-kDa truncated Ad12 E1A gene product. Virology 152: 375–383 (1986).
19. Kuppuswamy, M., Subramanian, T., and Chinnadurai, G. Separation of immortalization and T24-*ras* oncogene cooperative functions of adenovirus E1a. Oncogene 2: 613–615 (1988).
20. Spandidos, D. A., and Wilkie, N. M. Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature 310: 469–475 (1984).
21. Land, H., Chen, A. C., Morgenstern, J. P., Parada, L. F., and Weinberg, R. A. Behavior of *myc* and *ras* oncogenes in transformation of rat embryo fibroblasts. Mol. Cell. Biol. 6: 1917–1925 (1986).
22. Dotto, G. P., Parada, L. F., and Weinberg, R. F. Specific growth response of *ras*-transformed embryo fibroblasts to tumour promoters. Nature 318: 472–475 (1985).
23. Yamashita, T., Kato, H., and Fujinaga, K. Conditional immortalization and/or transformation of rat cells carrying v-*abl* or EJ*ras* oncogene in the presence or absence of glucocorticoid hormone. Int. J. Cancer 42: 930–938 (1988).
24. Dotto, G. P., Weinberg, R. A., and Ariza, A. Malignant transformation of mouse primary keratinocytes by Harvey sarcoma virus and its modulation by surrounding normal cells. Proc. Natl. Acad. Sci. U.S.A. 85: 6389–6393 (1988).
25. Franza, B. R., Maruyama, K., Garrels, J. I., and Ruley, H. E. *In vitro* establishment is not a sufficient prerequisite for transformation by activated *ras* oncogenes. 44: 409–418 (1986).
26. Lillie, J. W., Loewenstein, P. M., Green, M. R., and Green, M. Functional domains of adenovirus type 5 E1A proteins. Cell 50: 1091–1100 (1987).
27. Hirakawa, T., and Ruley, H. E. Rescue of cells from *ras* onco-

gene-induced growth arrest by a second, complementing oncogene. Proc. Natl. Acad. Sci. U.S.A. 85: 1519–1523 (1988).
28. McNeil, P. L., and Warder, E. Glass beads load macromolecules into living cells. J. Cell Sci. 88: 66–678 (1987).
29. Feramisco, J. R., Gross, M., Kamata, T., Rosenberg, M., and Sweet, R. W. Microinjection of the oncogene form of the human H-*ras* (T-24) protein results in rapid proliferation of quiescent cells. Cell 38: 109–117 (1984).
30. Stacey, D. W., and Kung, H. -F. Transformation of NIH 3T3 cells by microinjection of Ha-*ras* p21 protein. 310: 508–511 (1984).
31. Doolittle, R. F., Hunkapiller, M. W., Hood, L. E., DeVare, S. G., Robins, K. C., Aaronson, S. A., and Antoniades, H. N. Simian sarcoma virus *onc* gene, v-*sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221: 275–276 (1983).
32. Waterfield, M. D., Scrace, G. J., Whittle, N., Stroobant, P., Johnson, A., Wasteson, A., Westermark, B., Heldin, C. -H., Huang, J. S., and Deuel, T. F. Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304: 35–38 (1983).
33. Schreier, M., Kohler, G., Hengartner, H., Berek, C., Trucco, M., and Forni, L. Hybridoma Techniques—EMBO, SKMB Course. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1980.
34. Whyte, P., Williamson, N. M., and Harlow, E. Cellular targets for transformation by the adenovirus E1A proteins. Cell 56: 67–75 (1989).
35. Egan, C., Bayley, S. T., and Branton, P. E. Binding of the *Rb1* protein to E1A products is required for adenovirus transformation. Oncogene 4: 383–388 (1989).
36. Moran, E. A region of SV40 large T antigen can substitute for a transforming domain of the adenovirus E1A products. Nature 334: 168–170 (1988).
37. Ridley, A. J., Paterson, H. F., Noble, M., and Land, H. *Ras*-mediated cell cycle arrest is altered by nuclear oncogenes to induce cell transformation. EMBO J. 7: 1635–1645 (1988).
38. North, G. Regulating the cell cycle. Nature 339: 97–98 (1989).
39. Murray, A. W. The cell cycle as a cdc2 cycle. Nature 342: 14–15 (1989).
40. Draetta, G., Beach, D., and Moran, E. Synthesis of p34, the mammalian homolog of the yeast cdc2+/CDC28 protein kinase, is stimulated during adenovirus-induced proliferation of primary baby rat kidney cells. Oncogene 2: 553–557 (1988).
41. Giordano, E., Whyte, P., Harlow, E., Franza, B. R., Beach, D., and Draetta, G. A 60 kd cdc2-associated polypeptide complexes with the E1A proteins in adenovirus-infected cells. Cell 58: 981–990 (1989).
42. Josephs, S. F., Ratner, L., Clarke, M. F., Westin, E. H., Reitz, M. S., and Wong, S. F. Transforming potential of human c-*sis* nucleotide sequences encoding platelet-derived growth factor. Science 225: 636–639 (1984).
43. Blochlinger, K., and Diggelmann, H. Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. Mol. Cell. Biol. 4: 2929–2932 (1984).